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Role of Ethylene in the Senescence of Isolated Hibiscus Petals[1]

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ABSTRACT

Senescence of petals isolated from flowers of *Hibiscus rosa-sinensis* L. (cv Pink Versicolor) was associated with increased ethylene production. Exposure to ethylene (10 microliters per liter) accelerated the onset of senescence, as indicated by petal in-rolling, and stimulated ethylene production. Senescence was also hastened by basal application of 1-aminocyclopropane-1-carboxylic acid (ACC). Aminooxyacetic acid, an inhibitor of ethylene biosynthesis, effectively inhibited ethylene production by petals and delayed petal in-rolling. In marked contrast to these results with mature petals, immature petals isolated from flowers the day before flower opening did not respond to ethylene in terms of an increase in ethylene production or petal in-rolling. Furthermore, treatment with silver thiosulfate the day before flower opening effectively prevented petal senescence, while silver thiosulfate treatment on the morning of flower opening was ineffective. Application of ACC to both immature and mature petals greatly stimulated ethylene production indicating the presence of an active ethylene-forming enzyme in both tissues. Immature petals contained less free ACC than mature, presenescent petals and appeared to possess a more active system for converting ACC into its conjugated form. Thus, while the nature of the lack of responsiveness of immature petals to ethylene is unknown, ethylene production in hibiscus petals appears to be regulated by the control over ACC availability.

It is well established that ethylene is involved in the senescence of flowers (9). The role of ethylene in the aging process of carnations has been studied extensively (6, 8, 9, 16, 17, 20). In carnations, the onset of visible signs of senescence (*i.e.* petal wilting), or more subtle changes such as electrolyte leakage (20) occur concomitant with, or slightly after the climacteric peak in ethylene production. In this tissue, the increase in ethylene production has been implicated as a primary regulatory point in this developmental process.

It is apparent that ethylene biosynthesis is under tight metabolic control since its production is induced during certain stages of development such as senescence (23). Following the elucidation of the ethylene biosynthesis pathway by Adams and Yang (2), it was shown that the levels of ACC[3] increased in carnation petals concomitant with the increase in ethylene production (6). This indicated a possible regulatory role for ACC synthase, the enzyme responsible for the conversion of SAM to ACC, in carnation ethylene biosynthesis. In subsequent studies, it was found that the EFE, which converts ACC to ethylene, exhibited only minimal activity in preclimacteric carnation petals and increased during the aging process (8, 16, 22).

In contrast with the results with carnation flowers, ethylene was suggested as playing a less important role than initiating the senescence of the ephemeral flowers of *Ipomea* (13) *Tradescantia* (18). This was evidenced by the observations that in these flowers an increase in ethylene production commenced only after flower fading was evident. Since exposure of these flowers to exogenous ethylene induced senescene, it was concluded that ethylene accelerated a previously initiated response.

Trewavas (19) has argued that the sensitivity of plant tissue to hormones, rather than the concentration of the hormone, is the limiting factor in determining the plant's physiological or biochemical response. The sensitivity of flowers to ethylene varies greatly among plant species (9, 10). In general, there is a gradual increase in the flower's sensitivity to ethylene with increasing age (5, 9, 13, 18). The nature of this change in sensitivity is presently unknown.

The use of the ephemeral flowers of hibiscus offers an excellent model tissue for studying the developmental process of senescence. Their senescence is rapid and reproducible. Here, we describe the senescence of isolated hibiscus petals and show the relationship between tissue sensitivity to ethylene and ethylene biosynthesis. It was hoped that this research would begin to clarify some of the discrepancies which exist between the suggested role of ethylene in the senescence of carnations and ephemeral flowers.

MATERIALS AND METHODS

Plant Material. Flowers of hibiscus (*Hibiscus rosa-sinesis* L. cv Pink Versicolor) were harvested from plants grown outdoors on the Louisiana State University campus in Baton Rouge, LA. All experiments were conducted between June 1 and September 30, which is the peak period of flower production in this area. Flowers were harvested between 7:00 and 7:30 AM either on the morning of flower opening (d 0) or the morning of the day before opening (d −1) and immediately transported to the laboratory. Petals were carefully detached from the flowers at their bases. The petals' bases were then inserted through slits made in parafilm which was stetched across the top of 9-cm Petri dishes containing either glass distilled H_2O or treatment solutions. The petals were maintained in the laboratory under cool-white fluorescent light (1.5 W m^{-2}, 12-h d) at 22°C and approximately 60% RH. Five petals were placed in each Petri dish, and three replicate dishes were used for each treatment. Each experiment was repeated a minimum of twice.

Ethylene Measurement. Ethylene production was measured by sealing petals (five petals/replicate) in 0.25-L Mason jars fitted with sampling ports. Ethylene was allowed to accumulate for 1 h at which time 1-ml sample was withdrawn with a gas-tight syringe and anayzed for ethylene with a Hewlett-Packard gas

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[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; SAM, *S*-adenosylmethionine; EFE, Ethylene-forming enzyme; AOA, aminooxyacetic acid; STS, silver thiosulfate complex.

chromatograph model 5790 equipped with an Alumina column and a flame ionization detector.

Exposure to Exogenous Ethylene. Immediately following harvest, petals were detached from the flowers and placed with their bases in 10-ml distilled H_2O in a 0.25-L Mason jar. The jar was sealed and ethylene injected to yield a final concentration of 10 μl/L which was verified by GC. After 2 h of ethylene exposure, the petals were removed from the jar and placed with their bases in distilled H_2O as before.

Treatment with STS. The anionic STS was prepared according to the method of Reid *et al.* (17) with a final concentration of 4 mM Ag^+.

Extraction and Determination of Free and Conjugated ACC. Three 1-g replicate samples of petals were extracted with hot 80% ethanol using a glass homogenizer with a Teflon pestle. The resulting extract was centrifuged for 10 min at 10,000*g*. The pellet was resuspended in 80% ethanol and recentrifuged. The combined supernatants were evaporated to dryness under reduced pressure at 40°C. The dry residue was redissolved in 2 ml glass-distilled H_2O. An aliquot of this extract was assayed for free ACC by the method of Lizada and Yang (14). A second aliquot was made 2 N with respect to HCl and heated at 100°C for 3 h. The hydrolyzed extract was neutralized with NaOH and assayed for total ACC (free and acid labile) as before. Conjugated ACC (acid labile) was determined by subtracting free ACC from the total ACC.

RESULTS

Ethylene Production and Senescence. The senescence of hibiscus flowers was characterized by an in-rolling of the corolla followed by corolla wilting and abscission. Preliminary experiments showed petals isolated from flowers as early as 1 d before normal flower opening, opened and senesced in a manner and time sequence similar to intact flowers held under similar conditions. Given their outwardly visible indication of the onset of senescence (in-rolling) and their responsiveness to treatments, isolated petals were used to study hibiscus senescence.

Ethylene production by hibiscus petals was low for the first 8 h following flower opening, and then increased 15-fold over the next 16 h (Fig. 1). The beginning of petal in-rolling was apparent 12 h after flower opening at which time ethylene production had increased 7-fold. Ethylene production declined slightly between 24 and 32 h during which time the upper portions of the petals were visibly wilted. An increase in ACC occurred concomitantly with the rise in ethylene production associated with the onset of petal senescence (Fig. 1). Interestingly, the concentration of ACC continued to increase even after ethylene production by petals had begun to decline. This may be indicative of a loss in activity of the EFE.

Effect Ethylene, ACC, and AOA. Exposing petals to 10 μl/L ethylene for 2 h immediately following flower opening accelerated senescence as indicated by petal in-rolling (Fig. 2). This was associated with a substantial increase in ethylene production by petals. The pattern of ethylene evolution and petal in-rolling was similar to that of petals held in H_2O except for being advanced by at least 6 h. When petals were maintained in 0.1 mM ACC, ethylene production was stimulated 25-fold within 2 h of the application of ACC, indicating the solution was rapidly taken up and converted to ethylene (Fig. 3). This also accelerated petal senescence.

To test whether ethylene was acting by accelerating some previously initiated deteriorative changes or actually triggering the process, petals were held in a solution of 2 mM AOA, which inhibits ACC synthesis from SAM (23). Ethylene production by AOA-treated petals was not detected (level of sensitivity ≥ 0.010 μl/L) throughout the 32-h experiment (Fig. 3). Although senescence was delayed in AOA-treated petals, it was not prevented

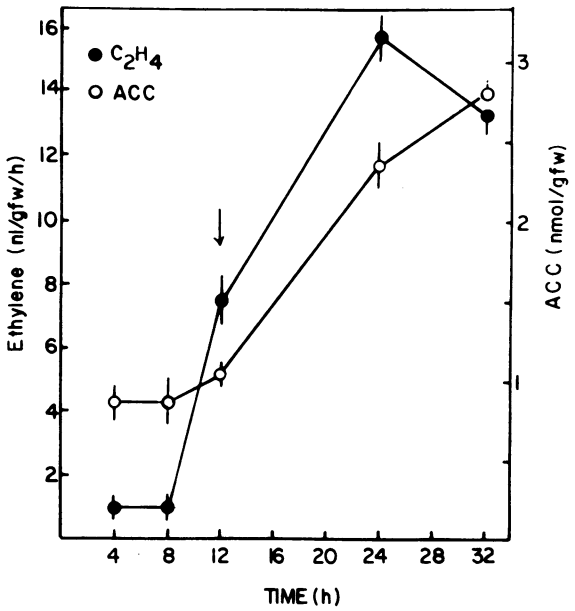

FIG. 1. Ethylene production and ACC content of isolated hibiscus petals during senescence. Petals were isolated from flowers at 8:00 the morning of flower opening and held in distilled H_2O between ethylene measurements. Three 1-g samples of petals each were selected at random for determination of ACC content at times indicated. Arrow indicates first visible sign of petal in-rolling. Means of three replications of five petals each. Vertical bars represent SE.

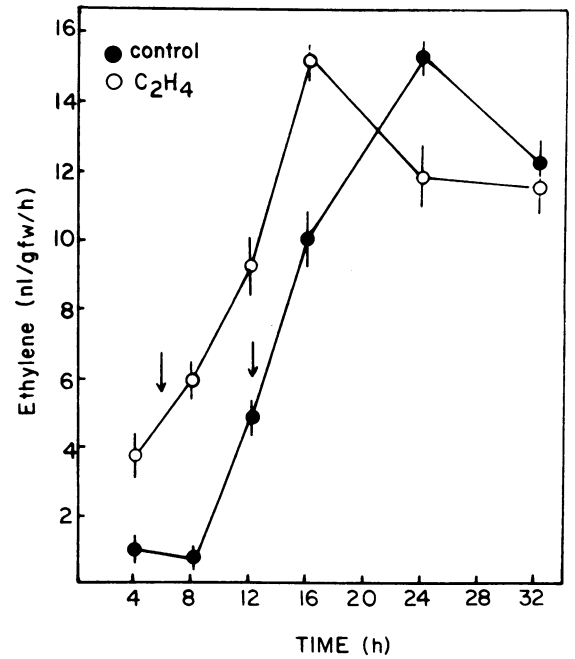

FIG. 2. Effect of exogenous ethylene on the senescence and ethylene production of isolated hibiscus petals. Petals were isolated from flowers at 8:00 the morning of flower opening and immediately exposed to 10 μl/L ethylene for 2 h. The petals were held with their bases in distilled H_2O during and after ethylene exposure. Ethylene production was determined at the times indicated after the start of ethylene treatment. Arrows indicate the first visible signs of petal in-rolling. Means of three replications of five petals each. Vertical bars represent SE.

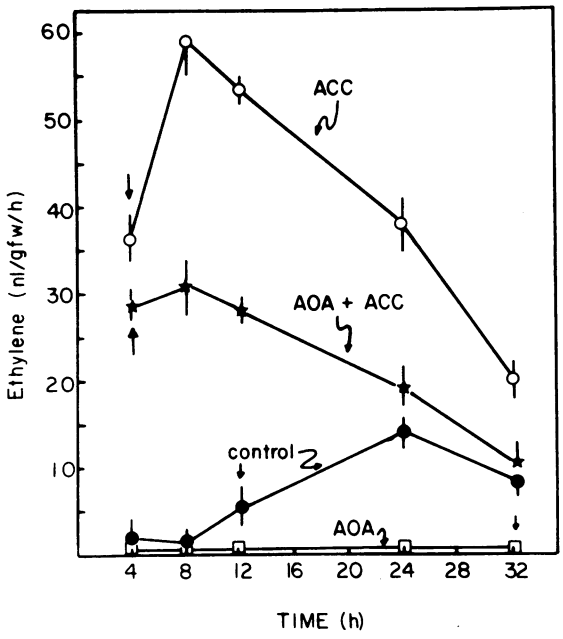

FIG. 3. Effect of exogenous ACC and AOA on the senescence and ethylene production of isolated hibiscus petals. Petals were isolated from flowers at 8:00 the morning of flower opening and placed with their bases in distilled H_2O, 0.1 mM ACC, 2 mM AOA, or ACC and AOA in combination. Ethylene production was determined at the times indicated after the start of treatments. Arrows indicate the first visible signs of petal in-rolling. Means of three replications of five petals each. Vertical bars represent SE.

since wilting and partial in-rolling were evident by the termination of the experiment. Application of ACC in combination with AOA reversed the inhibition of ethylene biosynthesis and accelerated senescence.

Effect of Petal Age. The response of flowers, as well as other organs, to ethylene is governed not only by the rate of ethylene synthesis, but also by the sensitivity of the tissue to ethylene (8, 9, 15). Therefore, it was of interest to determine the effects of exposure to ethylene or ACC on hibiscus petals of different physiological ages. Petals were isolated from intact flowers on the morning of d −1 or d 0. Immediately following harvest, petals were placed with their bases in 0.1 mM ACC or exposed to 10 μl/L ethylene as described under "Materials and Methods." After 2 h, petals were transferred to distilled H_2O for the remainder of the experiment. Both basal application of ACC and exposure to ethylene resulted in an acceleration of senescence and a substantial increase in ethylene production of petals harvested and treated on d 0 (Fig. 4). Petals isolated on the morning of d −1 senescenced on d 0 in a manner similar to those harvested on d 0. Exposure of d −1 petals to 10 μl/L ethylene did not accelerate senescence or stimulate an increase in ethylene production. Furthermore, the 2-h pulse treatment with 0.1 mM ACC did not promote senescence in spite of a brief stimulation of ethylene production.

A 2-h pulse treatment with 4 mM STS delayed the senescence of hibiscus petals on d 0 when applied on d −1 as determined by petal in-rolling and loss of fresh weight (Fig. 5). However, treatment with STS on the morning of d 0 failed to delay the onset of petal senescence.

Petal Age, EFE Activity, and ACC Conjugation. In an attempt to gain further insight into the regulation of ethylene synthesis in petals of different ages, we determined their capacity to convert exogenous ACC to ethylene or the inactive conjugated form. Petals isolated on d −1 and d 0 were held in 0.1 mM ACC for either 2 h immediately following harvest or throughout the 8-h experiment. Basal application of ACC substantially increased ethylene production in both immature (d −1) and mature (d 0) petals, indicating the presence of an active EFE in both tissues (Fig. 6). Petals isolated on d 0 showed visible signs of the onset of senescence 4 h after initiation of the ACC treatments. Ethylene production by d 0 petals held continuously in ACC peaked after 2 h, but remained high throughout the experiment. ACC continued to accumulate in these petals for 4 h, perhaps indicating a saturation of the EFE or loss of EFE activity with the progression of senescence. Petals isolated d −1 continued to increase in ethylene production and ACC content when held in ACC throughout the experiment without showing any visible signs of petal in-rolling or wilting.

When petals received a 2-h pulse treatment of ACC, the difference between the petals of different ages was striking. This brief exposure to ACC resulted in a substantial increase in ethylene production from both d 0 and d −1 petals. However, when petals were removed from ACC, ethylene production rapidly declined in d −1 petals while it remained high in d 0 petals. Furthermore, immature petals (d −1) showed a rapid decline in ACC content following removal from the exogenous ACC source, while mature petals (d 0) maintained an ACC concentration higher than those held in H_2O. Petals isolated on d −1 showed increased formation of the ACC conjugate, as compared to d 0 petals under both pulse and continuous ACC exposure. While ACC concentration rapidly declined in immature petals following a 2-h ACC pulse, the level of conjugated ACC increased.

DISCUSSION

The data reported here demonstrate clearly that senescence of hibiscus petals is associated with a substantial increase in ethylene production. Concomitant with the increase in ethylene production was an in-rolling of the petals similar to that observed in other flowers (9). Ethylene appears to be a regulator of hibiscus petal senescence as indicated by the in-rolling of the corolla. This view is based on the fact that exogenous ethylene or ACC accelerated petal senescence. Furthermore, AOA, which inhibits ACC synthesis from SAM, inhibited ethylene production and delayed petal senescence. The effects of AOA could be overcome by application of ACC.

As flowers mature, generally the system responsive to ethylene gradually becomes more sensitive (5, 8, 13, 18). Behavior of hibiscus petals showed a similar response. Petals could be isolated from hibiscus flowers the day before flower opening (d −1) and subsequently senesce on the following day (d 0) in a manner similar to petals isolated on the morning of flower opening. However, petals isolated on d −1 were completely insensitive to 10 μl/L exogenous ethylene. These petals exhibited no ethylene-induced in-rolling or increased ethylene production. In contrast, petals harvested on the morning of d 0 exhibited both responses to exogenous ethylene. The coupling between ethylene-induced in-rolling and ethylene synthesis varies with flower tissue used. Exposure of immature (d −1) *Ipomea* flowers to ethylene did result in in-rolling but not ethylene evolution, while mature *Ipomea* flowers exhibited both responses to exogenous ethylene (13).

Petal in-rolling is the visible end-result of more subtle cellular changes such as loss of membrane semipermeability. Since an increase in ethylene production by petals was not detected until in-rolling was apparent, it would be difficult to assign a senescence-initiating role to increased ethylene production. Rather, our data indicate metabolic events preceding the autocatalytic production of ethylene led to the senescence of hibiscus petals. This is in contrast with carnation petals, where increased ethylene synthesis appeared to be necessary for in-rolling (6, 7). Results similar to ours with other ephemeral flowers led previous re-

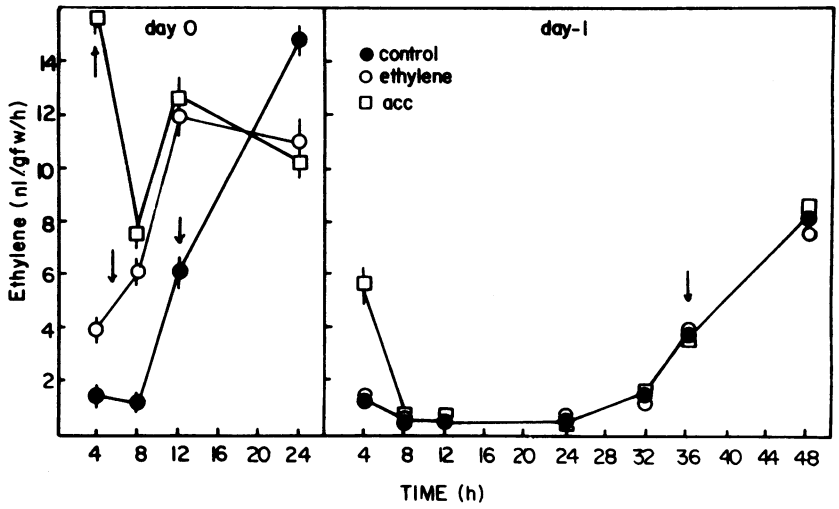

FIG. 4. Effect of exogenous ethylene and ACC on the senescence and ethylene production of hibiscus petals isolated from flowers of different ages. Petals were isolated from flowers at 8:00 the morning of flower opening (d 0) or the morning of the day before flower opening (d −1). After isolation, petals were immediately exposed to 10 μl/L ethylene for 2 h or held with their bases in 0.1 mM ACC for 2 h. Following ethylene exposure or ACC application, petals were transferred to distilled H_2O. Ethylene production was determined at the times indicated after the start of ethylene or ACC treatment. Arrows indicate the first visible signs of petal in-rolling. Means of three replications of five petals each. Vertical bars represent SE.

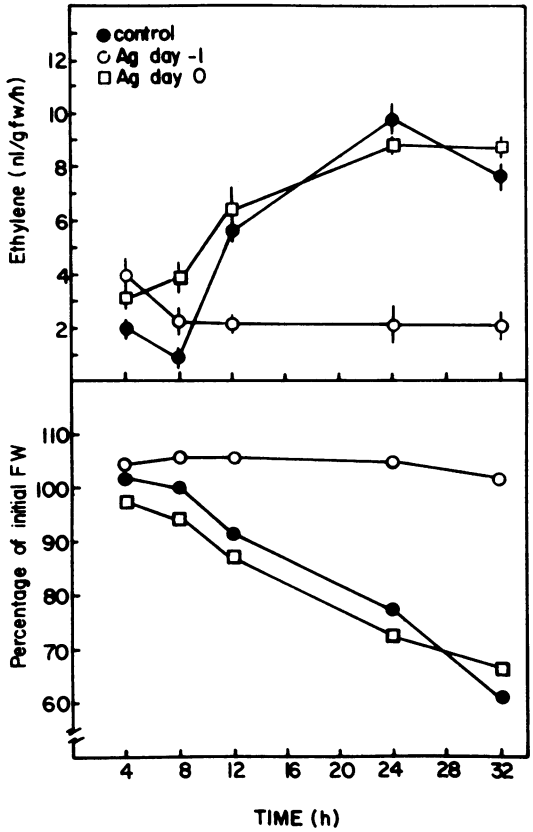

FIG. 5. Effect of STS on ethylene production and fresh weight loss of isolated hibiscus petals. Petals were isolated from intact flowers at 8:00 the morning before flower opening (d −1) and placed with their bases in distilled H_2O, or 4 mM STS for 2 h and then transferred to distilled H_2O. A second group of petals were transferred from distilled H_2O to 4 mM STS for 2 h the morning of d 0. Means of three replications of five petals each. Vertical bars represent SE.

searchers to conclude that ethylene accelerated a previously initiated event, rather than inducing the response (13, 18). It may be that the changing sensitivity to ethylene resulted in the petals responding to the low, preclimacteric ethylene levels in the tissue, thus regulating the onset of both in-rolling and ethylene production. Silver, applied as STS, which inhibits ethylene action (4, 17, 21), effectively prevented senescence when applied prior to flower opening, while delayed STS treatment had no effect (Fig. 5). This indicates that early events of senescence preceding in-rolling and autocatalytic ethylene production are mediated by ethylene.

The synthesis of ethylene in plant tissues is thought to be under tight metabolic control (23). Sites of control include ACC oxidation, ACC synthesis, and ACC conjugation. Application of exogenous ACC to plant tissues generally results in a marked increase in ethylene evolution (7), indicating the presence of an active EFE. In these tissues, the rate-limiting step in the synthesis of ethylene would appear to control the availability of ACC. Our data indicated that immature (d −1) as well as mature, preclimacteric hibiscus petals contain a very active EFE capable of rapidly converting applied ACC to ethylene. Much of our knowledge of ethylene-induced flower senescence was obtained using carnation (6, 8, 16, 22). In immature and preclimacteric carnation petals, the activity of the EFE is very low (16, 22), indicating the control of ethylene synthesis is at least partially mediated at the level of ACC oxidation to ethylene. In hibiscus petals, ethylene production appears to be limited by the availability of ACC, since any available ACC should be rapidly converted to ethylene. In general, this is attributed to a lack of ACC synthase activity (1, 7, 23). However, it is also possible that a separate storage and metabolic pool of either ACC or its precursors exists (8, 13).

Recently, the formation of an inactive conjugated form of ACC has been suggested as regulating ethylene biosynthesis in some tissues (3, 11, 12). Both immature (d −1) and mature hibiscus petals contained an acid labile conjugated from of ACC. We made no attempt to identify the conjugated form of ACC, but it has been identified in other tissues as 1-(malonylamino)cyclopropane-1-carboxylic acid (3, 11, 12). In senescing hibiscus petals, the concentration of conjugated ACC, while generally less than free ACC, followed the same pattern of increase as free ACC. It is interesting to note that petals isolated on d −1 seemed to possess a more active system for the conjugation of ACC. Indeed, in addition to conversion of exogenous ACC to ethylene, a substantial amount seemed to be diverted into the conjugated form. We found no evidence to suggest that the conjugated pool of ACC was subsequently used for ethylene synthesis since it remained high after ethylene production and ACC levels had dropped to that of the control. This is in

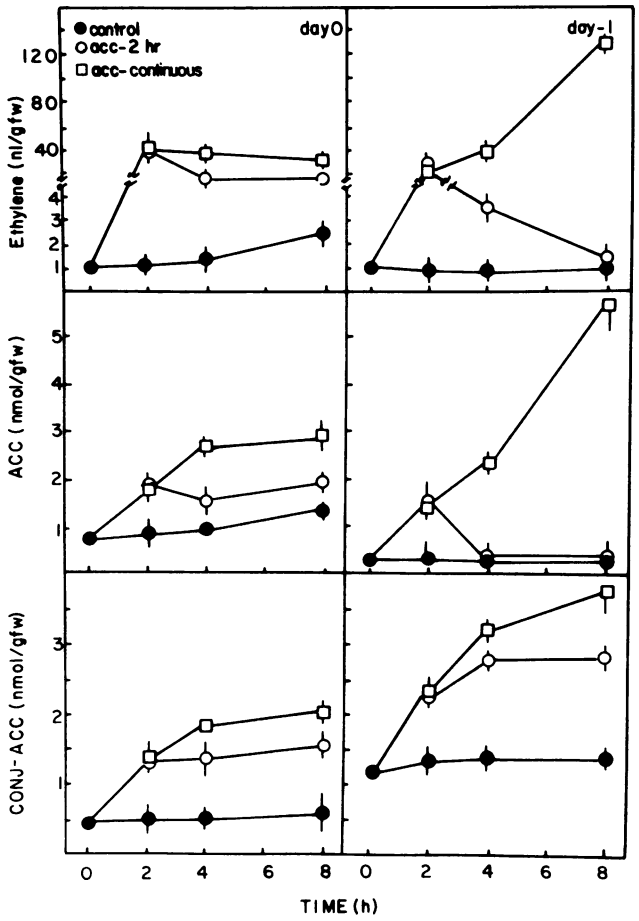

FIG. 6. Effect of exogenous ACC on the ethylene production and free and conjugated ACC contents of hibiscus petals of different ages. Petals were isolated from flowers at 8:00 the morning of flower opening (d 0) or the morning of the day before flower opening (d −1). After isolation petals were immediately placed with their bases in distilled H_2O or 0.1 mM ACC. Following 2 h in ACC, half the petals were transferred to distilled H_2O (ACC-pulse) while the remaining petals were kept in ACC for the entire experiment. At the times indicated after the start of ACC treatment, petals were selected at random for determination of ethylene production, and free conjugated ACC contents. Means of three replications of five petals each. Vertical bars represent SE.

agreement with other work which indicated that conjugated ACC is not appreciably metabolized (3, 11, 12). The conjugation of ACC in immature petals may serve as a regulatory mechanism to prevent the accumulation of free ACC.

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